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UTILITY PATENT APPLICATION TRANSMITTAL

(Only for new nonprovisional applications under 37 CFR 1.53(b))

Attorney Docket No.	99-041	Total Pages	39
First Named Inventor or Application Identifier	KEITH E. LEJEUNE ET AL.		
Express Mail Label No.	EL317713525US		

APPLICATION ELEMENTS

See MPEP chapter 600 concerning utility patent application contents.

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1. [X] Fee Transmittal Form *(Submit an original, and a duplicate for fee processing)*
2. [X] Specification [Total Pages 24] *(preferred arrangement set forth below)*
   - Descriptive title of the Invention
   - Cross References to Related Applications
   - Statement Regarding Fed sponsored R & D
   - Reference to Microfiche Appendix
   - Background of the Invention
   - Brief Summary of the Invention
   - Brief Description of the Drawings *(if filed)*
   - Detailed Description
   - Claim(s)
   - Abstract of the Disclosure
3. [X] Drawing(s) *(35 USC 113)* [Total Sheets 5]
4. Oath or Declaration [Total Pages 6]
   a. [X] Newly executed (original or copy)
   b. [ ] Copy from a prior application (37 CFR 1.63(d)) *(for continuation/divisional with Box 17 completed)* **[Note Box 5 below]**
      i. [ ] DELETION OF INVENTOR(S) Signed statement attached deleting inventor(s) named in the prior application, see 37 CFR 1.63(d)(2) and 1.33(b).
5. [ ] Incorporation By Reference *(useable if Box 4b is checked)* The entire disclosure of the prior application, from which a copy of the oath or declaration is supplied under Box 4b, is considered as being part of the disclosure of the accompanying application and is hereby incorporated by reference therein.
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ACCOMPANYING APPLICATION PARTS

8. [*] Assignment Papers (cover sheet & document(s))
9. [ ] 37 CFR 3.73(b) Statement *(when there is an assignee)* [ ] Power of Attorney
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11. [ ] Information Disclosure Statement (IDS)/PTO-1449 [ ] Copies of IDS Citations
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STATEMENT CLAIMING SMALL ENTITY STATUS (37 CFR 1.9(f) & 1.27(c))--SMALL BUSINESS CONCERN

Docket Number (Optional)
99-041

Applicant, Patentee, or Identifier: KEITH E. LEJEUNE AND ALAN J. RUSSELL
Application or Patent No.: TO BE ASSIGNED
Filed or Issued: NOVEMBER 17, 1999
Title: ENZYME-CONTAINING POLYURETHANES

I hereby state that I am
☐ the owner of the small business concern identified below:
☒ an official of the small business concern empowered to act on behalf of the concern identified below:

NAME OF SMALL BUSINESS CONCERN AGENTASE, LLC

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NAME OF PERSON SIGNING KEITH E. LEJEUNE

TITLE OF PERSON IF OTHER THAN OWNER PRESIDENT

ADDRESS OF PERSON SIGNING 300 TECHNOLOGY DRIVE, PITTSBURGH, PENNSYLVANIA USA

SIGNATURE _____ DATE _____

TITLE

ENZYME-CONTAINING POLYURETHANES

Field of the Invention

The present invention relates to enzyme-containing polyurethanes, and, especially, to enzyme-containing polyurethanes of relatively high enzyme loading and relatively high catalytic (enzyme) activity.

Background of the Invention

It has been known for some time that one can incorporate proteins within polyurethane polymers during polymer synthesis. For example, U.S. Patent Nos. 3,928,138, 3,929,574, 4,098,465, 4,195,127, and 4,250,267 describe enzymes bound within a hydrophilic polyurethane polymer. Although enzyme activity was evident in those polymers, no attempt was made to quantify the degree of enzyme activity within the polymers.

Academics have more recently begun to revisit the synthesis of enzymatic polyurethane. For example, Dias et al. assessed the performance of lipase incorporated within polyurethane foams. Dias, S. F., Vilas-Boas, L., Cabral, J. M. S., and Fonseca, M. M. R., Biocatalysis, 5, 21 (1991). That study described the synthesis of enzymatic polymers without the use of additives, enzyme stabilizers,

or enzyme pre-modification. Enzyme concentration within the polymers was varied over a broad range in the course of this study. Those studies indicated an apparent reduction in enzyme activity retention at high enzyme loading (for example, greater than 0.1 weight percent).

Storey et al described the immobilization of amyloglucosidase enzyme within several types of crosslinked polyurethane matrices Storey, K. B., Duncan, J. A., Chakrabarti, J. A., Appl. Biochem. Biotechnol. 23, 221 (1990). The enzyme concentrations employed in that study were relatively dilute and the use of additives or other non-essential components was not explored.

Recent studies of general polyurethane synthesis (irrespective of incorporation of enzyme therein) have shown that incorporation of a surfactant in the reaction mixture can lead to desirable physical properties of the polyurethane polymer product. It is believed that surfactants stabilize the carbon dioxide bubbles that are formed during synthesis and are responsible for foaming. For example, certain surfactants have been found to promote the creation of small carbon dioxide bubble, resulting in formation of a polymer product having a morphology similar to a fabric. Other surfactant have been found to promote relatively large carbon dioxide bubbles, resulting in a polymer product having a morphology similar to a sponge. Given the control that surfactants enable over the physical/morphological characteristics of polyurethanes, suppliers of polyurethane prepolymer typically recommend

that surfactant be added to a polyurethane reaction mixture.

Thus, recent studies of the synthesis of enzyme-containing polyurethanes have employed surfactants to alter/control the physical properties of the resultant polymers. For example, a number of studies describe the immobilization of organophosphorus hydrolase using a polyurethane polymer synthesis strategy in which a variety of non-ionic surfactants were used as additives to alter the physical properties polymers. Havens, P. L., Rase, H. F., Ind. Eng. Chem. Res., 32, 2254 (1993); LeJeune, K. E., Swers, J. S., Hetro, A. D., et al. Biotechnol. Bioeng., 64, 2, 250 (1999); LeJeune, K. E., et al. Biotechnol. Bioeng., 54, 105, (1997); LeJeune, K. E. and Russell, A. J. Biotechnol. Bioeng., 51, 450 (1996). In general, these surfactants were used in an attempt to optimize the performance of the polyurethane sponge product in a particular application. For example, the studies of Havens and Rase were focused upon using the resultant polymers as column packing material and as adsorbent sponges to decontaminate pesticide spills. The studies reported varying surfactant hydrophobicity could produce polymers that were better suited for a particular application. The enzyme concentration/loading employed in the studies of Havens and Rase and the other studies was quite low (in general, well below 0.1 weight percent of the polymer).

It is desirable to develop enzyme containing polymers and methods of synthesis of such polymers in which enzyme loading and enzyme activity are improved.

Summary of the Invention

The present inventors have discovered that certain surfactants not only enable control of polyurethane physical properties/morphology, but enhance the activity of immobilized enzymes at relatively high enzyme loading. As used herein, the term "enzyme" refers to a protein that catalyzes at least one biochemical reaction. A compound for which a particular enzyme catalyzes a reaction is typically referred to as a "substrate" of the enzyme. Enzymes typically have molecular weights in excess of 5000.

In general, six classes or types of enzymes (as classified by the type of reaction that is catalyzed) are recognized. Enzymes catalyzing reduction/oxidation or redox reactions are referred to generally as EC 1 (Enzyme Class 1) Oxidoreductases. Enzymes catalyzing the transfer of specific radicals or groups are referred to generally as EC 2 Transferases. Enzymes catalyzing hydrolysis are referred to generally as EC 3 hydrolases. Enzymes catalyzing removal from or addition to a substrate of specific chemical groups are referred to generally as EC 4 Lyases. Enzymes catalyzing isomeration are referred to generally as EC 5 Isomerases. Enzymes catalyzing combination or binding together of substrate units are referred to generally as EC 6 Ligases.

In one aspect, the present invention provides a method of increasing loading of active enzyme immobilized in a polyurethane polymer including the steps of:

synthesizing the polyurethane polymer in a reaction mixture containing water and enzyme; and

including a sufficient amount of a surfactant in the reaction mixture to increase enzyme activity at an enzyme loading (as compared to a polymer of the same enzyme loading synthesized without surfactant).

As used herein, the term "surfactant: refers generally to a surface active agent that is reduces the surface tension of a liquid (water, for example) in which it is dissolved.

Preferably, the surfactant is nonionic and comprises between 0.5 to 5.0 weight percent of the aqueous component of the mixture. In the synthesis of the polyurethanes of the present invention, urethane prepolymers were mixed with water. The aqueous component of the reaction mixture included water, enzyme, surfactant and buffer salts. The weight percent surfactant in the aqueous component is thus calculated by dividing the weight of the surfactant by the weight of the entire aqueous component and multiplying the result by 100%. The enzyme loading in the present invention can be greater than approximately 0.1 percent by weight of the polyurethane polymer (weight of enzyme/[weight of enzyme-containing polymer product]*100%) while retaining substantial enzyme activity. Relatively high activity is maintained even when the enzyme loading is greater that approximately 0.5 percent by weight of the polyurethane polymer. Indeed, relatively high activity is maintained even when the enzyme

loading is greater that approximately 1 percent by weight of the polyurethane polymer.

The polyurethane polymers of the present invention preferably include at least one of an oxidoreductase, a transferase, a hydrolase, a lyase, an isomerase or a ligase. Examples of enzymes suitable for use in the present invention include, but are not limited to, a lipase, a peroxidase, a tyrosinase, a glycosidase, a nuclease, a aldolase, a phosphatase, a sulfatase, or a dehydrogenase.

More than one type of enzyme are easily co-immobilized within the polyurethane polymer. The enzymes can be within the same class (for example, two hydrolases) or a within different classes of enzyme.

In another aspect, the present invention provides a polyurethane polymer containing an enzyme loading of more than approximately 0.1 weight percent. The polyurethane polymer is synthesized in the presence of a sufficient amount of a surfactant (preferably, nonionic) to increase enzyme activity at the enzyme loading of the polymer (as compared to the case when no surfactant is used).

In still another aspect, the present invention provides a method of improving enzymatic activity of a polyurethane polymer synthesized with an enzyme loading of more than approximately 0.1 weight percent. The method includes the step of:

adding a sufficient amount of a surfactant (preferably, nonionic) to during synthesis of the polyurethane polymer to increase enzyme activity at the enzyme loading.

The polymers and methods of the present invention provide enhanced enzyme activity retention as the enzyme loading or enzyme content of such polymers is increased (for example, to above approximately 0.1 weight percent of the polymer). Relatively large quantities of enzymes are immobilized within the polymers of the present invention while retaining a significant portion of the native enzyme specific activity.

Brief Description of the Drawings

Figure 1 illustrates an embodiment of a synthetic scheme synthesis of enzyme-containing polymers.

Figure 2 illustrates the effect of using surfactant in the synthesis of subtilisin-containing polyurethane polymers.

Figure 3 illustrates the effect of using surfactant in the synthesis of urease-containing polyurethane polymers.

Figure 4 illustrates the effect of surfactant concentration upon the catalytic activity of polymer containing subtilisin carlsberg.

Figure 5 illustrates a study of the utility of non-ionic surfactants in polymer synthesis as compared to surfactants that are cationic or anionic in nature.

Detailed Description of the Invention

The enzyme-containing polyurethane polymers of the present invention can, for example, be synthesized by reaction of relatively hydrophilic polyurethane prepolymer with aqueous solution to produce a urethane foam. The polyurethane prepolymers used in the present studies were urethanes that were capped (that is, functionalized at chain ends) with multiple isocyanate functionalities. Prepolymers containing multiple isocyanate functionalities have the ability to form chemical crosslinks upon reaction with a diol or water. Water reacts with isocyanates, initiating a foaming reaction in which a carbamic acid intermediate is formed. The carbamic acid quickly degrades to an amine and evolves CO_2. The carbon dioxide bubbles through the highly viscous reacting polymer solution, creating a porous foam structure. Because amines readily react with isocyanates, a multi-functional prepolymer in aqueous solution results in a crosslinked polyurethane matrix.

Because the vast majority of enzymes are most active in aqueous solution, water not only serves to initiate the prepolymeric reaction, but also provides a route to deliver an enzyme to the reaction. Proteins such as enzymes have many amine groups present via lysine

residues and can readily react with isocyanate functionalities to form a crosslinked polymer-protein network through multi-point attachments of the enzyme and polymer. A schematic of the reactions occurring in this process is illustrated in Figure 1. In Figure 1, R_1 represents a prepolymer molecule (for example, having a molecular weight of approximately 300 to approximately 10,000) having multiple isocyanate functionalities/groups. R_2 and R represent other prepolymer molecules with isocyanate functionalities. E represents an enzyme with a reactive amine functionality present via lysine residues and at the N-terminus of the protein.

It is believed that the surfactants used in synthesis of enzyme-containing polyurethane polymers of the present invention enhance the activity of biocatalytic polymers when the enzymatic content of the composite materials is sufficiently high to overwhelm the capacity of the polymer to provide the enzyme incorporated therein with sufficient access to bind substrate or to release product at a rate equivalent to the maximum achievable catalytic rate. In that regard, several studies of the present invention have demonstrated that polymers with excessive enzyme content are diffusionally limited in their ability to catalyze reactions. It is believed, that the use of certain surfactants over a range of concentrations eliminates the diffusional limitations imposed by a polymeric superstructure within which relatively large amounts of enzyme have been incorporated.

EXPERIMENTAL PROCEDURES

1. Enzyme polymer synthesis

As known in the art, variation of reaction conditions affects both the physical properties polyurethane foams and the degree of enzyme-foam interaction. Described below is a typical procedure for biopolymer synthesis used in the present studies. Initially, 4 ml of pH 7.8 Tris buffer (10 mM) containing a specific surfactant at a particular concentration (approximately 0 to 8 weight percent in the studies of the present invention) were placed into a narrow cylindrical mixing vessel. Subsequently, an enzyme solution (for example approximately 1 ml of 1.5 mg/ml urease in the same buffer, for example) was added. Finally, approximately 4 ml of Hypol prepolymer, available from Hampshire Chemical Corp., a subsidiary of Dow Chemical Company, (preheated to 30°C to limit handling problems resulting from high viscosity) were added to the mixture. The solutions was then intimately mixed. During the initial "cream" period, the solution was injected into a cylindrical mold where it rose and then set within 2 to 5 minutes. Polymer synthesis was complete in less than 10 minutes. The CO_2 evolved during the reaction of water and isocyanate lifted the foam to a final volume of approximately 50 to 60 ml.

After the initial 10 minute "set-up" time, foam samples were treated in several ways. Some foam samples were immediately sealed in vials, while others were pre-rinsed. Bulk foam samples were typically placed in a fume

hood or lyophilizer to facilitate the removal of residual water and CO_2 still present from the reaction. Foams were stored under a wide range of conditions until being assayed for enzyme activity.

The mixing system used in the present studies required 30 to 40 seconds of mixing at 2500 rpm to create a high quality foam with Hypol 3000, a toluene di-isocyanate based prepolymer. The mixing system included an oar-shaped metal loop having a height of 3.2 cm and a diameter of 1.3 cm. Hypol 5000 (methylene bis(p-phenyl isocyanate) based), a more hydrophobic prepolymer, required additional mixing. Insufficient mixing can result in un-reacted residual prepolymer dispersed within a dense hard mass of polyurethane. Overmixing does not allow the evolving CO_2 to act in lifting the foam. Properly mixed foam will typically increase approximately six-fold in volume throughout the course of the reaction.

In one embodiment of the present invention, an aqueous solution of enzymes and surfactant was contacted with an isocyanate-based prepolymer under sufficient agitation to initiate reaction. The enzyme can, for example, be added as a freeze-dried powder or aqueous solution that is either pure or impure. The term "impure" a used herein refers generally to enzymes containing, for example, other proteins/enzymes and biological molecules. Virtually any enzyme or combination of enzymes can be co-immobilized within the same polymer in the present invention.

In model studies of the present invention, enzyme-containing polymers were synthesized both with and without a series of surfactants. Enzymes incorporated into the polymers of the present invention included, for example, organophosphorus hydrolase (OPH), organophosphorus acid anhydrolase (OPAA), butyrylcholinesterase (BChE), urease, and subtilisin carlsberg. The benefit of using certain surfactants in the synthesis of the enzyme-containing polymers of the present invention was demonstrated with in series of kinetic experiments discussed below.

2. Increasing enzyme activity in highly loaded polymers through the use of surfactants

Using the procedures described above for polymer synthesis, enzyme-containing polymers were synthesized both with and without the use of surfactants. For example, subtilisin carlsberg and urease enzymes were individually incorporated within polyurethane polymers over a range of enzyme concentrations from approximately 10μg to approximately 20mg enzyme per gram polymer (that is, approximately .001 to approximately 2% by weight. Multiple polymers were synthesized at each enzyme concentration, some with the use of 1 weight percent Pluronic F-68 non-ionic surfactant present and some without surfactant. The polymers were placed in a fume hood for 12 hours after synthesis to facilitate the removal of residual water and CO_2 before their catalytic activity was assessed.

Subtilisin-containing polymers were assayed for their hydrolytic activity on N-succinyl-ALA-ALA-PRO-PHE p-

nitroanilide in 10% MeOH/50mM Tris buffer (pH 8.0) solutions. Substrate hydrolysis was monitored with the use of a spectrophotometer. Reaction rates were determined by placing polymer samples (100mg) within 10ml substrate solutions and taking and subsequently replacing aliquots from the reacting system at regular intervals. Figure 2 illustrates the benefit achieved by including surfactant within the polymer formulation. Without the addition of surfactants during the polymer synthesis, very little if any benefit is incurred by increasing the enzyme content within the polymers, whereas those polymers synthesized in the presence of surfactant exhibited activity levels which were closely related to enzyme content.

The activity of urease polymers (150 mg samples) was assayed in 300mM urea within 10mM Phosphate buffer at pH 7.25 (15ml). Urea hydrolysis was assessed by monitoring solution pH, since urea hydrolysis causes a corresponding increase in pH. Figure 3 shows that there are no significant diffusional limitations present at low enzyme concentration. The rates of reaction with or without surfactant are essentially identical when the urease content of the polymer is low (see Table 1 for rate data). The rate of catalysis is proportional to enzyme concentration in the presence of surfactant. However, the absence of surfactant is believed to result in diffusional limitations within the system. Apparent catalytic activity was found to have very little dependence upon enzyme loading when surfactants are not utilized in polymer synthesis.

Table 1. Rate data for urease-polymer assays.

	Polymers synthesized with surfactant	**Polymers synthesized without surfactant**
Enzyme loading in polymer (μg urease / g polymer)	Reaction Rate (ΔpH / min)*10^3	Reaction Rate (ΔpH / min)*10^3
170	8.2	6.4
430	15.0	8.7
1700	63.0	9.8

Diffusional limitations for other enzymes (including organophosphorus hydrolase, organophosphorus acid anhydrolase, and butyrylcholinesterase) have also been measured.

3. Surfactant concentration

The amount of surfactant present during polymer synthesis was found to affect the retention of enzyme activity in the enzyme-containing polymers of the present invention. In the limit as surfactant concentration approaches zero, the resulting material exhibits the same polymer properties and subsequent diffusional limitations present when no surfactant is employed. The effect of the amount of surfactant used was studied by synthesizing biocatalytic polymers with sufficient enzyme loading to cause diffusional limitation in the absence of surfactant. Nearly identical synthetic procedures were also carried out for polymers in which Pluronic F-68 surfactant content was gradually increased to near its solubility limit in water (7.5%).

Subtilisin-containing polymers were synthesized with an enzyme loading of approximately 250µg enzyme per gram polymer. This degree of loading is believed to be sufficient to incur diffusional limitations within polymers not formulated for high enzyme loading (see Figure 2) through the use of sufficient surfactant during synthesis. Figure 4 illustrates a study of the effect of surfactant concentration upon the rate of catalysis observed for subtilisin-polymers (100mg) having an enzyme loading of 250µg enzyme per gram polymer that were assayed against N-succinyl-ALA-ALA-PRO-PHE p-nitroanilide in 10% MeOH / 50 mM Tris buffer (pH 8.0) solutions (10 ml). Surfactant concentrations of less than 0.5 weight percent of the aqueous synthesis component were found to be insufficient to overcome the diffusional limitations imposed at this level of enzyme loading. The data indicate that an "optimum" surfactant concentration exists between approximately 0.5 and approximately 5.0 weight percent. The data of Table 2 indicates that increasing surfactant concentration beyond the optimum concentration did not further improve activity. It is possible that diffusional limitations were overcome at the enzyme loading studied at the lower surfactant concentration.

Table 2. Rate data for urease-polymer assays.

Surfactant concentration used in polymer synthesis (wt% aqueous phase)	**Observed Rate** (ΔAbs_{400nm} / min)*10^2
0.01	1.5
0.1	2.7
0.5	7.7
2.5	9.1
5.0	8.8
7.5	7.9

4. The nature of the surfactant

The above studies demonstrated that retention of enzyme activity is improved through the use of surfactants in polymer synthesis. There are, however, many types of surfactants which one might envision using to synthesize polymers. The broadest classification of surfactants is based upon the charge of the head group. The available surfactant pool includes of anionic, cationic, and non-ionic surfactants. In several studies, two representative surfactants were selected from each group and employed in polymer synthesis at a loading of 1 weight percent of the aqueous component of the synthesis mixture. Polymers without surfactant and without enzyme were synthesized as controls for the activity assays.

Subtilisin was used as a model enzyme in these studies. The procedures described above were employed to synthesize the enzyme polymers (200μg subtilisin/gram polymer). Anionic (lauryl sulfate, octyl sulfate), cationic (cetylpyridinium chloride,

dodecyltrimethylammonium bromide) and non-ionic (Pluronic™ F-68 available from BASF Corp., Mount Olive, New Jersey and Tween™ 20 available from ICI Americas, Wilmington Delaware) surfactants were each used in synthesizing individual enzyme polymer samples. The polymers were exposed to open air in a fume hood for several hours before assay to facilitate the removal of residual water and CO_2.

The resulting polymers were assayed for their hydrolytic activity on N-succinyl-ALA-ALA-PRO-PHE p-nitroanilide in 10% MeOH/50mM Tris buffer (pH 8.0) solutions. Substrate hydrolysis was monitored with the use of a spectrophotometer at 400nm. Reaction rates were determined by placing polymer samples (100mg) within 10ml substrate solutions. The data of Figure 5 indicate that, while polymers synthesized without surfactant or with cationic or anionic surfactants have appreciable catalytic activity (compared to the corresponding polymer without enzyme), one preferably uses non-ionic surfactant(s) in polymer synthesis to maximize the retained activity of the enzyme immobilized therein. Table 3 further illustrates this phenomenon. The relative reaction rates are ratios based upon the catalytic rates achieved when no surfactant is employed.

Table 3. Relative catalytic rates when employing different surfactants in subtilisin polymer synthesis.

Surfactant Classification	**Surfactant used in polymer synthesis** (1 wt% of aqueous phase)	**Relative Reaction Rates**
	None	1.0
Anionic	Lauryl sulfate	1.0
	Octyl sulfate	1.1

Cationic	Cetylpyridinium chloride	1.5
	Dodecyltrimethylammonium bromide	1.3
Non-ionic	Pluronic F-68	5.8
	Tween 20	6.2

Although the present invention has been described in detail in connection with the above examples, it is to be understood that such detail is solely for that purpose and that variations can be made by those skilled in the art without departing from the spirit of the invention except as it may be limited by the following claims.

WHAT IS CLAIMED IS:

1. A method of increasing loading of active enzyme immobilized in a polyurethane polymer, the method comprising the steps of:

synthesizing the polyurethane polymer in a reaction mixture containing water and enzyme; and

including a sufficient amount of a surfactant in the reaction mixture to increase enzyme activity at an enzyme loading.

2. The method of Claim 1 wherein the surfactant is nonionic and the enzyme loading is greater that approximately 0.1 percent by weight of the polyurethane polymer.

3. The method of Claim 1 wherein the enzyme loading is greater that approximately 0.5 percent by weight of the polyurethane polymer.

4. The method of Claim 1 wherein the enzyme loading is greater that approximately 1 percent by weight of the polyurethane polymer.

5. The method of Claim 2 wherein enzyme immobilized in the polyurethane polymer includes at least one of an oxidoreductase, a transferase, a hydrolase, a lyase, an isomerase or a ligase.

6. The method of Claim 2 wherein enzyme immobilized in the polyurethane polymer includes at least one of a protease, a lipase, a peroxidase, a tyrosinase, a glycosidase, a nuclease, a aldolase, a phosphatase, a sulfatase, or a dehydrogenase.

7. The method of Claim 2 wherein at least two species of enzyme are co-immobilized within the polyurethane polymer.

8. The method of Claim 7 wherein the two species of enzyme are within the same class of enzyme.

9. The method of Claim 2 wherein the surfactant comprises between 0.5 to 5.0 weight percent of the aqueous component of the mixture.

10. The method of Claim 1 wherein the enzyme is a hydrolase and the surfactant is nonionic.

11. A polyurethane polymer containing an enzyme loading of more than approximately 0.1 weight percent enzyme, the polyurethane polymer having been synthesized in the presence of a sufficient amount of a surfactant to increase enzyme activity at the enzyme loading.

12. The polyurethane polymer of Claim 11 wherein the surfactant is nonionic.

13. The polyurethane polymer of Claim 12 wherein the enzyme loading is greater that approximately 0.5 percent by weight of the polyurethane polymer.

14. The polyurethane polymer of Claim 12 wherein the enzyme loading is greater that approximately 1 percent by weight of the polyurethane polymer.

15. The polyurethane polymer of Claim 12 wherein enzyme immobilized in the polyurethane polymer includes at least one of an oxidoreductase, a transferase, a proteolytic enzyme, a lyase, an isomerase or a ligase.

16. The polyurethane polymer of Claim 12 wherein enzyme immobilized in the polyurethane polymer includes at least one of a protease, a lipase, a peroxidase, a tyrosinase, a glycosidase, a nuclease, a aldolase, a phosphatase, a sulfatase, a hydrolase, or a dehydrogenase.

17. The polyurethane polymer of Claim 12 wherein at least two species of enzyme are co-immobilized within the polyurethane polymer.

18. The polyurethane polymer of Claim 17 wherein the two species of enzyme are within the same class of enzyme.

19. The polyurethane polymer of Claim 12 wherein the surfactant comprises between 0.5 to 5.0 weight percent of the aqueous component of a reaction mixture.

20. The polyurethane polymer of Claim 12 wherein the enzyme is a hydrolase and the surfactant is nonionic.

21. A method of improving enzymatic activity in a polyurethane polymer synthesized with an enzyme loading of more than approximately 0.1 weight percent enzyme, the method comprising the step of:

adding a sufficient amount of a surfactant during synthesis of the polyurethane polymer to increase enzyme activity at the enzyme loading.

22. The method of Claim 21 wherein the surfactant is nonionic.

23. The method of Claim 22 wherein the enzyme loading is greater that approximately 0.5 percent by weight of the polyurethane polymer.

24. The method of Claim 22 wherein the enzyme loading is greater that approximately 1 percent by weight of the polyurethane polymer.

25. The method of Claim 22 wherein enzyme immobilized in the polyurethane polymer includes at least one of an oxidoreductase, a transferase, a proteolytic enzyme, a lyase, an isomerase or a ligase.

26. The method of Claim 22 wherein enzyme immobilized in the polyurethane polymer includes at least

one of a protease, a lipase, a peroxidase, a tyrosinase, a glycosidase, a nuclease, a aldolase, a phosphatase, a sulfatase, a hydrolase, or a dehydrogenase.

27. The method of Claim 22 wherein at least two species of enzyme are co-immobilized within the polyurethane polymer.

28. The method of Claim 27 wherein the two species of enzyme are within the same class of enzyme.

29. The method of Claim 22 wherein the surfactant comprises between 0.5 to 5.0 weight percent of the aqueous component of the mixture.

30. The method of Claim 22 wherein the enzyme is a hydrolase and surfactant is nonionic.

ABSTRACT

A method of increasing loading of active enzyme immobilized in a polyurethane polymer including the steps of: synthesizing the polyurethane polymer in a reaction mixture containing water and enzyme; and including a sufficient amount of a surfactant in the reaction mixture to increase enzyme activity at an enzyme loading.

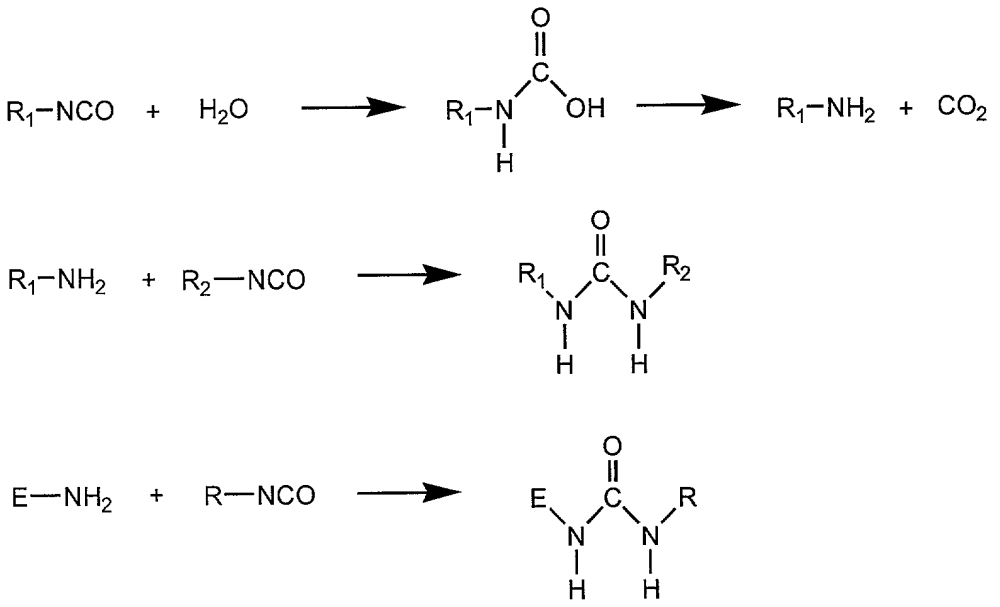

Figure 1. Reaction schematic of biopolymer synthesis.

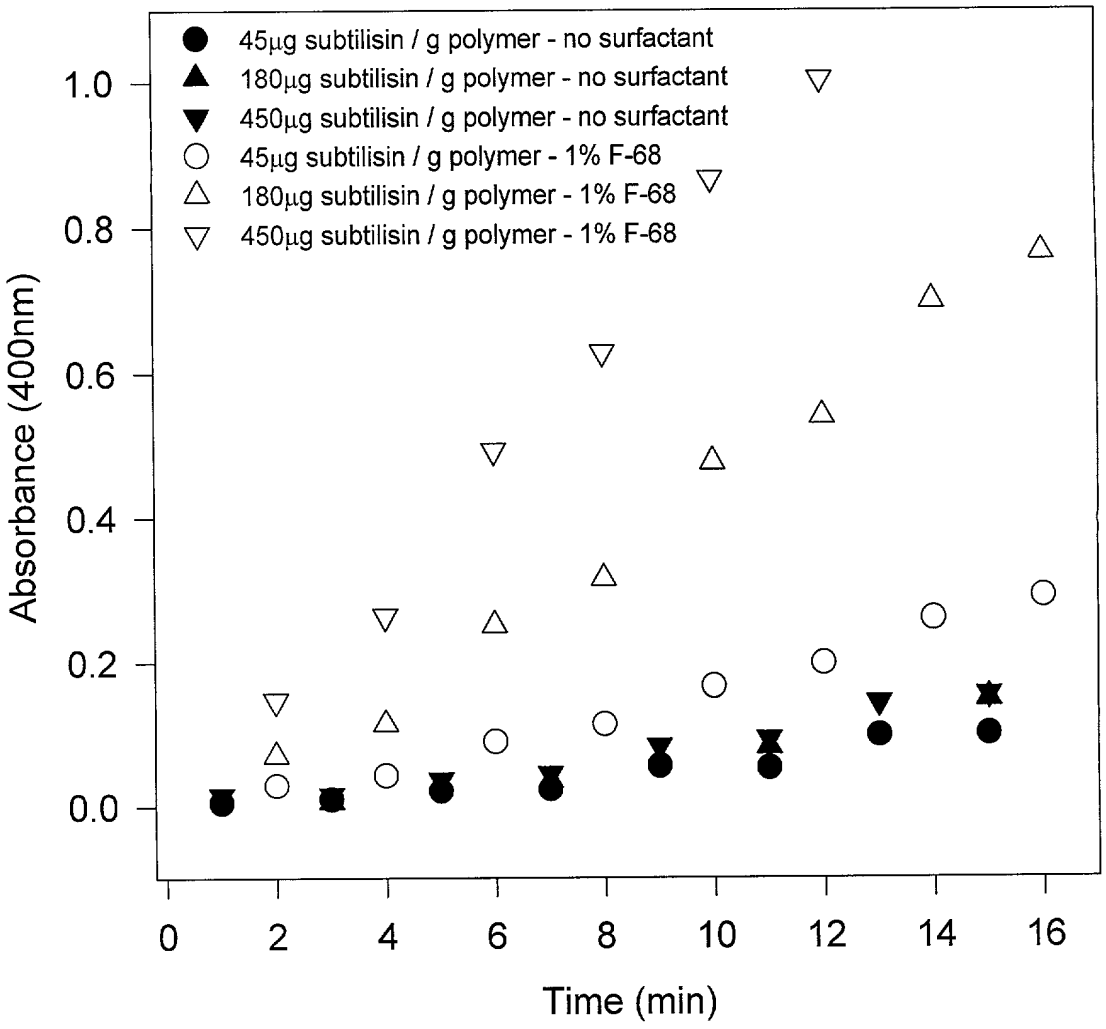

Figure 2. Surfactant effect on catalytic activity of subtilsin polymers.

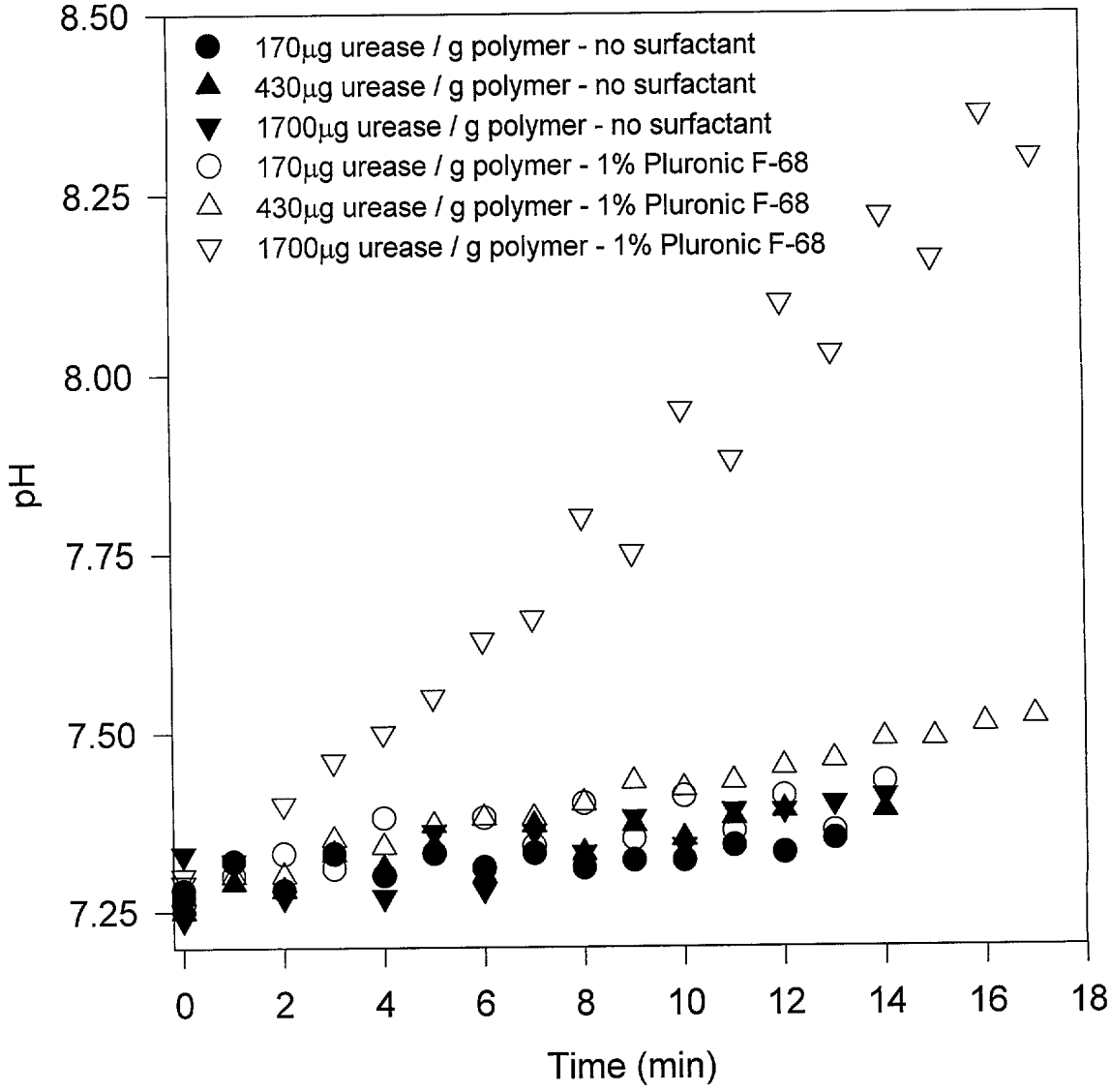

Figure 3. Surfactant effects on activity of urease polymers.

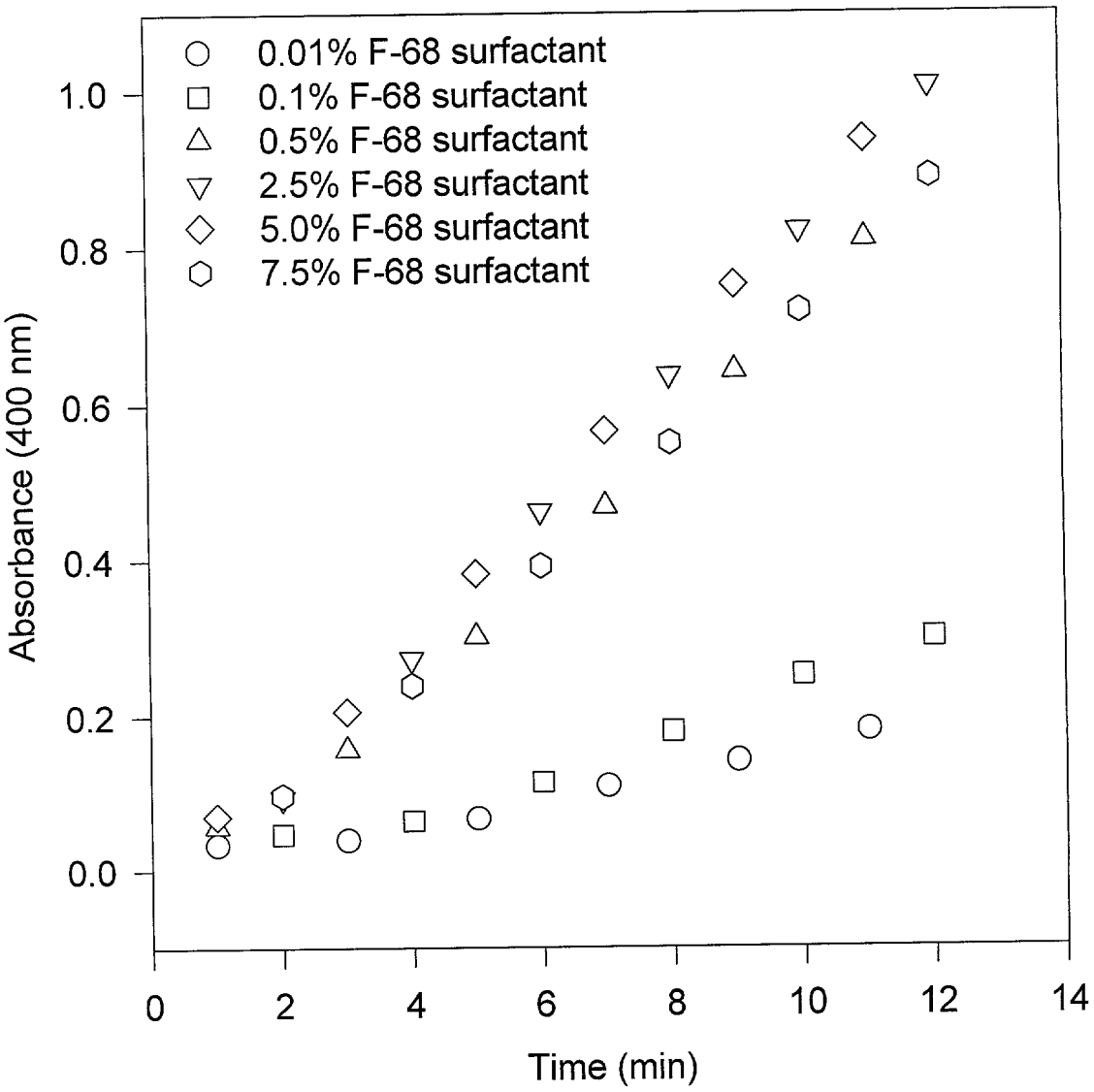

Figure 4. Effect of F-68 surfactant concentration on subtilisin-polymer activity.

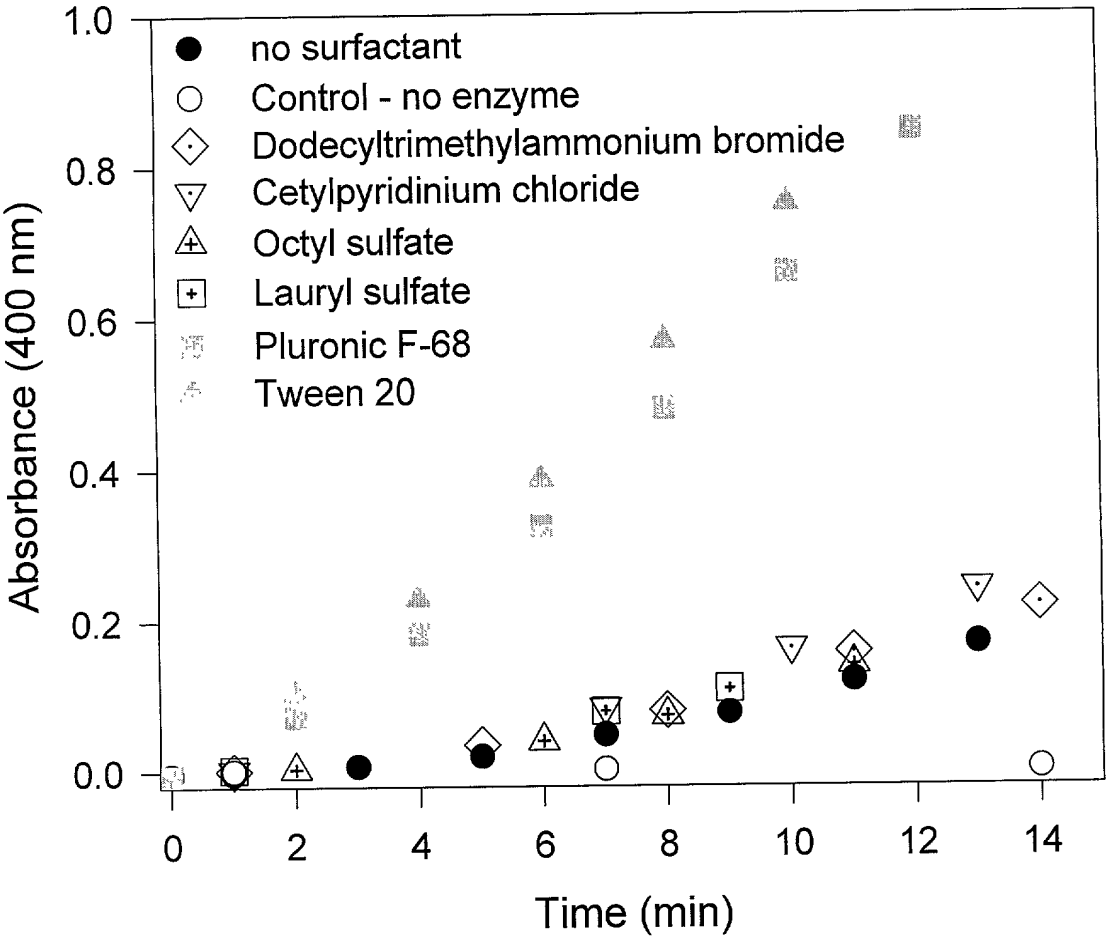

Figure 5. Effect of using different surfactants on subtilisin-polymer activity.

Attorney's Docket No. 99-041 PATENT

COMBINED DECLARATION AND POWER OF ATTORNEY

(ORIGINAL, DESIGN, NATIONAL STAGE OF PCT, SUPPLEMENTAL, DIVISIONAL, CONTINUATION OR C-I-P)

As a below named inventor, I hereby declare that:

TYPE OF DECLARATION

This declaration is of the following type:

(check one applicable item below)

[x] original.

[ ] design.

[ ] supplemental.

NOTE: IF THE DECLARATION IS FOR AN INTERNATIONAL APPLICATION BEING FILED AS A DIVISIONAL, CONTINUATION OR CONTINUATION-IN-PART APPLICATION, DO NOT CHECK NEXT ITEM; CHECK APPROPRIATE ONE OF LAST THREE ITEMS.

[ ] national stage of PCT.

NOTE: IF ONE OF THE FOLLOWING 3 ITEMS APPLY, THEN COMPLETE AND ALSO ATTACH ADDED PAGES FOR DIVISIONAL, CONTINUATION OR C-I-P.

[ ] divisional.

[ ] continuation.

[ ] continuation-in-part (C-I-P).

INVENTORSHIP IDENTIFICATION

WARNING: IF THE INVENTORS ARE EACH NOT THE INVENTORS OF ALL THE CLAIMS, AN EXPLANATION OF THE FACTS, INCLUDING THE OWNERSHIP OF ALL THE CLAIMS AT THE TIME THE LAST CLAIMED INVENTION WAS MADE, SHOULD BE SUBMITTED.

My residence, post office address and citizenship are as stated below, next to my name. I believe that I am the original, first and sole inventor (IF ONLY ONE NAME IS LISTED BELOW) or an original, first and joint inventor (IF PLURAL NAMES ARE LISTED BELOW) of the subject matter that is claimed, and for which a patent is sought on the invention entitled:

TITLE OF INVENTION

ENZYME-CONTAINING POLYURETHANES

SPECIFICATION IDENTIFICATION

the specification of which:

(complete (a), (b) or (c))

(a) [x] is attached hereto.

(b) [ ] was filed on _____, as [ ] Serial No. _____
or [ ] Express Mail No. _____, as Serial No. not yet known
and was amended on _____(IF APPLICABLE).

NOTE: AMENDMENTS FILED AFTER THE ORIGINAL PAPERS ARE DEPOSITED WITH THE PTO THAT CONTAIN NEW MATTER ARE NOT ACCORDED A FILING DATE BY BEING REFERRED TO IN THE DECLARATION. ACCORDINGLY, THE AMENDMENTS INVOLVED ARE THOSE FILED WITH THE APPLICATION PAPERS OR, IN THE CASE OF A SUPPLEMENTAL DECLARATION, ARE THOSE AMENDMENTS CLAIMING MATTER NOT ENCOMPASSED IN THE ORIGINAL STATEMENT OF INVENTION OR CLAIMS. SEE 37 CFR 1.67.

(c) [ ] was described and claimed in PCT International Application No. _____, filed on _____and as amended under PCT Article 19 on _____(IF ANY).

ACKNOWLEDGEMENT OF REVIEW OF PAPERS AND DUTY OF CANDOR

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above.

I acknowledge the duty to disclose information, which is material to patentability as defined in 37, Code of Federal Regulations, S 1.56,

(also check the following items, if desired)

[x] and which is material to the examination of this application, namely, information where there is a substantial likelihood that a reasonable Examiner would consider it important in deciding whether to allow the application to issue as a patent, and

[ ] in compliance with this duty, there is attached an information disclosure statement, in accordance with 37 CFR 1.98.

PRIORITY CLAIM (35 U.S.C. S 119(a)-(d))

I hereby claim foreign priority benefits under Title 35, United States Code, S 119(a)-(d) of any foreign application(s) for patent or inventor's certificate or of any PCT international application(s) designating at least one country other than the United States of America listed below and have also identified below any foreign application(s) for patent or inventor's certificate or any PCT international application(s) designating at least one country other than the United States of America filed by me on the same subject matter having a filing date before that of the application(s) of which priority is claimed.

(complete (d) or (e))

(d) [x] no such applications have been filed.

(e) [ ] such applications have been filed as follows.

NOTE: WHERE ITEM (C) IS ENTERED ABOVE AND THE INTERNATIONAL APPLICATION WHICH DESIGNATED THE U.S. ITSELF CLAIMED PRIORITY CHECK ITEM (E), ENTER THE DETAILS BELOW AND MAKE THE PRIORITY CLAIM.

PRIOR FOREIGN/PCT APPLICATION(S) FILED WITHIN 12 MONTHS (6 MONTHS FOR DESIGN) PRIOR TO THIS APPLICATION AND ANY PRIORITY CLAIMS UNDER 35 U.S.C. S 119(a)-(d)

COUNTRY (OR INDICATE IF PCT)	APPLICATION NUMBER	DATE OF FILING (day, month, year)	PRIORITY CLAIMED UNDER 37 USC 119
			[ ] YES NO [ ]
			[ ] YES NO [ ]
			[ ] YES NO [ ]
			[ ] YES NO [ ]
			[ ] YES NO [ ]

CLAIM FOR BENEFIT OF PRIOR U.S. PROVISIONAL APPLICATION(S) (34 U.S.C. S 119(e))

I hereby claim the benefit under Title 35, United States Code, S 119(e) of any United States provisional application(s) listed below:

PROVISIONAL APPLICATION NUMBER	FILING DATE
_____/_____	_____
_____/_____	_____
_____/_____	_____

CLAIM FOR BENEFIT OF EARLIER US/PCT APPLICATION(S) UNDER 35 U.S.C. 120

[ ] The claim for the benefit of any such applications are set forth in the attached ADDED PAGES TO COMBINED DECLARATION AND POWER OF ATTORNEY FOR DIVISIONAL, CONTINUATION OR CONTINUATION-IN PART (C-I-P) APPLICATION.

ALL FOREIGN APPLICATION(S), IF ANY, FILED MORE THAN 12 MONTHS (6 MONTHS FOR DESIGN) PRIOR TO THIS U.S. APPLICATION

NOTE: IF THE APPLICATION FILED MORE THAN 12 MONTHS FROM THE FILING DATE OF THIS APPLICATION IS A PCT FILING FORMING THE BASIS FOR THIS APPLICATION ENTERING THE UNITED STATES AS (1) THE NATIONAL STAGE, OR (2) A CONTINUATION, DIVISIONAL, OR CONTINUATION-IN-PART, THEN ALSO COMPLETE ADDED PAGES TO COMBINED DECLARATION AND POWER OF ATTORNEY FOR DIVISIONAL, CONTINUATION OR C-I-P APPLICATION FOR BENEFIT OF THE PRIOR U.S. OR PCT APPLICATION(S) UNDER 35 U.S.C. S 120.

POWER OF ATTORNEY

I hereby appoint the following attorney(s) and/or agent(s) to prosecute this application and transact all business in the Patent and Trademark Office connected therewith.

Henry E. Bartony, Jr., Reg. No. 34,772

(check the following item, if applicable)

[ ] Attached, as part of this declaration and power of attorney, is the authorization of the above-named attorney(s) to accept and follow instructions from my representative(s).

SEND CORRESPONDENCE TO

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DIRECT TELEPHONE CALLS TO:

Henry E. Bartony, Jr.

(412) 338-8632

DECLARATION

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

SIGNATURE(S)

NOTE: Carefully indicate the family (or last) name, as it should appear on the filing receipt and all other documents.

Full name of sole or first inventor

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*****

Full name of third joint inventor, if any

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Inventor's signature ____________________

Date ____________ Country of Citizenship USA

Residence ____________________

Post Office Address ____________________

(check proper box(es) for any of the following added page(s) that form a part of this declaration)

[ ] Signature for fourth and subsequent joint inventors. NUMBER OF PAGES ADDED _____.

* * *

[ ] Signature by administrator(trix), executor(trix) or legal representative for deceased or incapacitated inventor. NUMBER OF PAGES ADDED _____.

* * *

[ ] Signature for inventor who refuses to sign or cannot be reached by person authorized under 37 CFR 1.47. NUMBER OF PAGES ADDED _____.

* * *

[ ] Added page for signature by one joint inventor on behalf of deceased inventor(s) where legal representative cannot be appointed in time. (37 CFR 1.47)

* * *

[ ] Added pages to combined declaration and power of attorney for divisional, continuation, or continuation-in-part (C-I-P) application.

[x] Total Number of pages added _____

* * *

[ ] Authorization of attorney(s) to accept and follow instructions from representative.

* * *

(if no further pages form a part of this Declaration, then end this Declaration with this page and check the following item)

[x] This declaration ends with this page.